HIGH-THROUGHPUT 3-D CELLULAR ASSAYS USING DESTABILIZED GREEN FLUORESCENCE PROTEIN

A THESIS

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By

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ABSTRACT

Cell assays for high-throughput screening (HTS) of potential drug candidates are important tools in the process of drug discovery. Most cellular assays are currently based on 2-D monolayer cell cultures, but 3-D cell cultures could better mimic the *in vivo* characteristics of actual organism tissues. Unfortunately, assays using 3-D culture models usually require significant manual manipulation and are therefore not suitable for HTS. Research under Dr. Shang-Tian Yang has resulted in a functioning system for high-throughput 3-D cellular assays using engineered cells to express enhanced green fluorescence protein (EGFP) quantifiable through fluorometry. System improvement to allow rapid assessment of cellular events, such as specific gene expression or cell cycle progress may be limited by the relatively long persistence of the currently used reporter protein in the cells.

In this study a new fluorescence reporting cell line was established using a destabilized EGFP (d4EGFP) expressed in Chinese hamster ovary (CHO) cells. Correlating the fluorescence response with cell number for the d4EGFP expressing cell line in 2-D assays indicated that the fluorescence expression of d4EGFP may be too low for use in reporting cell number in a high-throughput manner. The fluorescence and cell number correlation in 3-D assays indicated that slightly better performance could be achieved with the d4EGFP reporter in 3-D but further testing is needed to demonstrate whether this improvement would be sufficient. Future work investigating growth and environmental conditions or further genetic modification of the cell line is recommended to possibly improve the fluorescence expression.

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Chapter 1. Introduction

High-throughput screening (HTS) has become an important tool for drug discovery. The number of potential drug candidates is rapidly increasing and HTS allows for the testing of a large amount of potential drug candidates in a short amount of time. HTS systems that provide faster and better predictions on the merits of potential candidates will provide improved efficiency and lower costs for the discovery of new drugs.

Cellular assay tests are one popular type of HTS but most current cellular assay tests are done on a 2-D monolayer cell culture. However, 2-D cellular assays can produce misleading results since much of the functionality of *in vivo* tissues is lost when using a simple 2-D culture. A solution to this problem is to use 3-D cellular assays which are more similar to the actual environment of cells. Unlike in 2-D assays, effects including cellular aggregation, diffusion limitation, and differential gene expression can be present in 3-D cellular assays and thus are a better approximation of *in vivo* conditions (Mueller-Klieser, 1997).

Optical reporting using internally expressed reporter proteins such as enhanced green fluorescence protein (EGFP) is a popular approach for quantifying cellular changes in assay tests. Such optical reporting systems can assess cell number in 3-D without disrupting the growth and are not adversely affected by layering of cells. To assess rapid cellular responses in high-throughput, including changes in protein expression or promoter activity, more sensitive optical reporting is needed. Destabilized EGFP (dEGFP) is more rapidly degraded in mammalian cells and thus there is more turnover of

fluorescence protein in the cell which could provide the sensitivity in response needed to improve HTS cellular assays.

This research focused on the development of a d4EGFP ($t_{1/2}$ = 4 hrs) expressing Chinese hamster ovary (CHO) cell line. The expression of d4EGFP was validated through fluorescence microscopy and the ability of the fluorescence to quantify cell number. Changes in cell number was assessed for the d4EGFP expressing cells and compared to EGFP expressing cells previously developed by the group. Correlation between cell number and fluorescence was assessed in a 2D monolayer culture and in a culture using 3D scaffolds. Fluorescence response over extended cell growth in 2-D was compared with cell number change over the growth period. The effect of toxic agents on fluorescence response was also be assessed. The results showed that fluorescence response can be correlated with cell number for d4EGFP expressing cells, though the fluorescence intensity per cell was lower than desired in 2-D culture and might not allow for accurate determination of changes in cell growth without requiring manual manipulation and disturbing cell growth. Recommendations are made on improving fluorescence response and future work is also outlined for assessing the suitability of the reporter protein for 3-D assays in this thesis.

Chapter 2. Literature Review

2.1 Biological assays for HTS

Biomolecular assays can provide detailed information on the reaction kinetics of specific biomolecular interactions (VanWiggeren et al., 2007), but how those interactions ultimately affect living organisms can be quite complicated. *In vitro* cellular assays can show the overall effect of a particular agent even if some of the specificity of the biomolecular assay is gone. Even so, many cellular assay systems fail to capture the interactions of different tissues and dynamic changes and even lack many of the features of tissues in the body. *In vivo* animal models can account for the effects of an agent on an entire organism but animal testing is expensive, lacks high-throughput capability, can raise moral concerns and still does not produce the same results as the human body. Ultimately all of these steps are necessary in the process of identifying new effective drug agents, but improvement of earlier less expensive steps can ease the burden of the drug discovery process. One of the shortcomings of cellular assays is due to the fact that most current cellular assay tests are done on a 2-D monolayer cell culture. Unfortunately, since much of the functionality of *in vivo* tissues is lost when using a simple 2-D culture, these types of assays often produce misleading results. Drugs may be effective on a 2-D monolayer but ineffective *in vivo* due to a variety of reasons such as poor penetration into solid tissues or differences in native cell morphology and metabolism (Mueller-Klieser, 1997). Much money is wasted when successful candidates in 2-D studies are advanced to more expensive *in vivo* animal testing and fail due to differences between 2-D and *in vivo* cellular characteristics (Seiler et al, 2003).

A solution to this problem is to use 3-D cellular assays which are more similar to the actual environment of cells. Unlike in 2-D assays, effects including cellular aggregation, diffusion limitation, and differential gene expression can be present in 3-D cellular assays and thus are a better approximation of *in vivo* conditions (Mueller-Klieser, 1997). However, one serious drawback to 3-D assays is that they usually require a significant amount of manual manipulation and consequently are not well suited for HTS.

2.2 Green fluorescence protein (GFP) based optical reporting

It is difficult to assess cells number in 3-D without disrupting the growth. Optical reporting methods using internally expressed reporter proteins can offer a solution to this problem. One such reporter protein is green fluorescence protein (GFP). GFP was originally isolated from *Aequorea* jellyfish by Shimomura et al. (1962) and is now a popular reporter protein for biological research (Arun et al., 2005). Unlike other optical reporter systems currently available such as luciferase and β-galactosidase, GFP does not require the addition of substrates which reduces the manual manipulation involved and improves the potential for high-throughput application (Yang et al., 1993; Zhang et al. 1996). GFP fluoresces naturally due to the light-emitting chromophore within the primary amino acid sequence (Arun et al. 2005). The chromophore is a cyclic tripeptide consisting of Ser-Tyr-Gly at positions 65-67 and requires a cyclization reaction and an oxidation step at the Tyr66 to form (Arun et al., 2005). The chromophore is contained in a central α-helix enclosed in a tightly packed β-can structure (Arun et al., 2005). Amino acids 7-229 of the 238 amino-acid protein are necessary for fluorescence (Arun et al., 2005). A number of variants to wild-type GFP (wt-GFP) have been developed which

broaden the uses of GFP. Red-shifted variants including enhanced green fluorescence protein (EGFP) have an excitation peak shifted closer to the wavelength emitted by lasers commonly used with fluorescence-activated cell sorting (FACS) devices and confocal scanning laser microscopes (Arun et al., 2005). EGFP also features a 4-35 fold increase in fluorescence intensity as compared to wt-GFP, due to a double amino acid substitution [Phe-64 to Leu and Ser-65 to Thr] (Arun et al., 2005). Both the shift to a more desirable excitation peak as well as the increased fluorescence make EGFP highly suited for use as a reporter protein for cellular assays.

2.3 Successful HTS 3-D cell assay system using EGFP

Our research group has made significant strides in the development and optimization of a HTS 3-D cellular assay system (Zhang et al. 2005; Zhang, Wen et al. 2007; Zhang, Ng et al. 2007, Zhang, 2008). A HTS system using genetically modified embryonic stem (ES) cells expressing EGFP cultured in 3-D fibrous scaffolds was developed. This system only required manual manipulation at initiation and cell number can be determined automatically using fluorometry through the whole experiment due to the stable expression of EGFP. This system has been shown to effectively monitor cell growth kinetics, and has produced cytotoxicity results consistent with other results in literature and clinical trials (Zhang, 2008; Smitskamp-Wilms et al., 1998). Planned improvement for the system includes extending the system to be able to assess rapid cellular responses. However, the current fluorescence protein being used (EGFP) has a half life of over one day and may not be well suited for this purpose as changes in fluorescence may not occur fast enough.

2.4 Current work with destabilized EGFP

As discussed by Kain (1999) variants of GFP called destabilized EGFP (dEGFP) have been engineered to by fusing a PEST amino acid sequence, enriched in proline (P), glutamic acid (E), serine (S) and threonine (T), to the C-terminus of EGFP (Rechsteiner, 1996). As a result of the PEST sequence proteolysis of the protein by a 26S proteosome is expedited in mammalian cells. Variants d1EGFP, d2EGFP and d4EGPF exist, formed through mutagenesis of the PEST sequence, and have half lives of one, two and four hours respectively, as compared to over 24 hrs for EGFP. Though the half lives are different, the spectral properties of the dEGFP variants are the same as for EGFP since amino acids 7-229 which are needed for fluorescence remain unchanged from EGFP. These variants have been used as an alternative to EGFP when changes in protein expression or promoter activity are variables of interest. (Kain, 1999)

Because dEGFP is more rapidly degraded there is more turnover of fluorescence protein in the cell. When cells are growing, increases in fluorescence should be more closely associated with the change in cell number and not the accumulation of fluorescence protein in existing cells. When cells are exposed to a toxic agent that reduces protein expression or kills the cell, the fluorescence response should be faster since accumulated protein will be degraded more quickly.

The dEGFP variants have already been used for their more sensitive response. Proteasome activity has been monitored in a mouse neuroblastoma cell line using a constitutively expressed d2EGFP reporter by Nahreini et al. (2003). Significant increases in fluorescence were observed in response to proteasome inhibition after 24 hrs as determined from flow cytometry (Nahreini et al., 2003). The d4EGFP variant has also

been used to test the transcriptional activity of synthetic mammalian promoters for HiB5, ARPE-19, and CD8+ T cell lines (Tornøe et al., 2002; Cooper et al., 2004). After cell transfection with d4EGFP under the control of various promoters, differences in fluorescence levels as determined using flow cytometry were used to indicate the relative transcriptional activity of the different promoters. Though these works support the use of dEGFP for specific protein and transcription activity analysis, they do not feature the use of dEGFP in high-throughput since flow cytometry requires sample preparation and disturbs cell growth.

The d2EGFP variant has also been used to monitor specific gene expression pathway activity (Ullerås et al., 2005). A d2EGFP reporting system with the reporter under the control of a cytokine promoter in mouse immune system cells has shown d2EGFP-mediated fluorescence change in a dose dependent fashion following exposure to xenobiotics to induce or inhibit cytokine expression as determined using fluorescence microscopy and flow cytometry (Ullerås et al., 2005). Khan et al. (2006) used an expression vector encoding an in-frame fusion of d4EGFP with the human Bid protein, a pro-apoptotic protein, to monitor protein translocation using fluorescence microscopy. Localized intracellular fluorescence was seen in images from microscopy indicating where the Bid protein resides in the cell (Khan et al., 2006). King et al. (2007) studied dynamic, promoter induced changes in protein expression using d4EGFP and d2EGFP under the control of various inducible promoters. Fluorescence was monitored automatically every 90 minutes using quantitative fluorescence microscopy and the resultant fluorescence profiles had reasonable consistency with expected promoter induction characteristics (King et al., 2007). The use of fluorescence microscopy in

assessing fluorescence response has more potential for high-throughput capability as it is not necessary to disturb cell growth, but is expensive if quantitative results are desired and cannot provide rapid results for a large number of samples (Haney et al., 2006).

Hansen et al. (2002) have used d2EGFP under the control of a NF-κB transcription factor to show redox-sensitive transcription changes. In their system, fluorometry was used to quantify fluorescence but only after centrifugation and freeze/thawing of the cells (Hansen et al., 2002). Though the fluorescence response was measured using fluorometry, which is highly suited for high-throughput applications, manual sample preparation and steps disturbing cell growth were still performed and it is unclear whether suitable fluorescence response could have been obtained directly without such manipulation which would be more desirable for high-throughput.

Chapter 3. Materials and Methods

3.1 Cell maintenance

CHO cells were maintained in T-flasks using a 1:1 ratio of Ham's F-12 Medium and Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 5% fetal bovine serum (FBS, Gibco), 5% CDM4CHO, 1% glutamine, and 0.2% MTX.

3.2 Transfection and clonal isolation

The plasmid vector pd4EGFP-N1 (Clontech, Palo Alto, CA) was used which features the d4EGFP gene under the control of a human cytomegalovirus (CMV) promoter, a strong constitutive promoter with expression levels not varying much due to environmental effects. The plasmid was transfected into CHO cells using Lipofectamine (Invitrogen). To perform the transfection the cells were plated into a 12 well plate and allowed to grow to roughly 70% confluence. At that point the regular growth medium was replaced with 500 μL FBS free medium and incubated for 1 hour. Lipofectamine and the plasmid were added to separate containers of 500 μL FBS free medium for a final concentration of 2 μg/ml for each. The Lipofectamine and plasmid solutions were combined after 5 minutes and then pipetted over the cells after 20 minutes. The medium was then replaced with growth medium after 4-6 hours. Once cells had reached confluence, further amplification was done in a small t-flask. When cells had reached 70% confluence, fluorescence positive cells were selected using FACSCalibur (BD, CA). Cells were amplified again and then cultured in a 96 well plate (BD Optilux^{TM}, Black/clear bottom) at a concentration of 1-2 cells per well. After amplification, wells

with a single fluorescent colony and no non-fluorescent colonies were selected using a fluorescence microscope. Those cell lines were amplified and put in cold storage in 5% DMSO, 40% FBS, and 55% growth medium.

3.3 Fluorescence signal and cell number correlation in 2-D

For both d4EGFP and EGFP expressing cell lines cells were amplified in several 25-cm^2 T-flasks then trypsinized, centrifuged and re-suspended in growth medium. Cell concentration was determined with a hemocytomter and known amounts of cells (0, 0.5, 1, 2, and 4 million cells) were added to wells of a 96 well plate and suspended in 180 μL of growth medium. Fluorescence intensity was then measured with a Cytofluor Series 4000 from Applied Biosystems, Foster City, CA (excitation at 485nm with a bandwidth of 20 nm and emission at 530 nm with a bandwidth of 25 nm).

3.4 Fluorescence signal and cell number correlation in 3-D

Following the method of Zhang (2008), 3D culture scaffolds were made from hole punched polyethylene terephtalate (PET) fabric (fiber diameter, ~20 μm; fiber density 1.35 g/cm^3, 0.1 cm height, 0.3 cm^2 bottom surface area). Scaffolds were added to a solution of 1% (w/v) Na_2CO_3 and 1% (v/v) Tween-20, heated to 60^oC and left to soak for 30 min. Scaffolds were rinsed with distilled water and then boiled in a 1% NaOH solution for 30 min. After being washed again scaffolds were soaked in PBS and sterilized in an autoclave at 121^oC for at least 20 minutes. The sterilized scaffolds were then stored at room temperature prior to use. Before use scaffolds were soaked in 10 μg/ml fibronectin for 12 hours and then in growth medium for 12 hours. Growth medium

was removed and for both d4EGFP and EGFP expressing cells 25 µl of known amount of cells (0.06, 0.12, 0.25 and 0.50 million cells) were seeded onto each scaffold. After 6 hours, 180 µL of medium was added to each plate and the fluorescence was measured.

3.5 Growth kinetics

EGFP and d4EGFP expressing cells were plated in multiple 25 cm^2 T-flasks, 6 well plates, as well as in a 96 well plate. Over 5 days fluorescence measurements were taken from the 3 sources and cells were counted from wells of the 96 well plate using a hemocytometer. For each sample, the cells from one T-flask for d4EGFP and one well of the 6 well plates for d4EGFP and EGFP were removed and suspended in 180 µL of either medium or PBS in the 96 well plate. For both the d4EGFP and the EGFP cell lines one of the wells in the 96 well plate had its medium replaced with PBS and then the fluorescence of all wells was measured. Afterwards, a sample was taken from one of the wells for EGFP and d4EGFP and the number of cells was counted using a hemocytometer.

3.6 2-D toxicity in PBS suspension

EGFP and d4EGFP expressing cells were added to a 96 well plate suspended in 180 µL of PBS at a concentration of 0.5 million cells per well. Either etoposide (Sigma) or doxorubicin (Sigma) was added to wells at a final concentration of either 10 µg/mL or 50 µg/mL. The fluorescence signal was measured continuously over 4 hours.

3.7 2-D toxicity with anchored cells

Ten thousand d4EGFP expressing cells were added to wells of a 96 well plate in 180 μL of growth medium and incubated for 12 hours. Drug was added to wells at a final concentration of 50 μg/mL. The drugs used were etoposide, doxorubicin, and 5-fluorouracil (Sigma). The fluorescence signal was measured continuously over 4 hours.

Chapter 4. Results and Discussion

4.1 Transfection and clonal isolation

The plasmid transfection efficiency was consistently around 10% as determined via fluorescence microscopy. After allowing the cells to amplify for until close to 100% confluence was achieved in a 25-cm^2 T-flask, the percentage of cells with detectable fluorescence had decreased to roughly 1% possibly indicating that genomic integration of the plasmid had not occurred in a majority of the positively transfected cells. Cells were sorted using FASC with 0.8% positive events. Cells were amplified again and around 10% of cells had positive fluorescence; however, the intensity of fluorescence was less than immediately after transfection. After plating 1-2 cells in each well of a 96 well plate and amplifying for 10 days wells were selected that had a single fluorescence-positive colony and no fluorescence-negative colonies. The cells in wells meeting these criteria were amplified and determined to be stably fluorescing after many passages by fluorescence microscopy (see Figure 1).

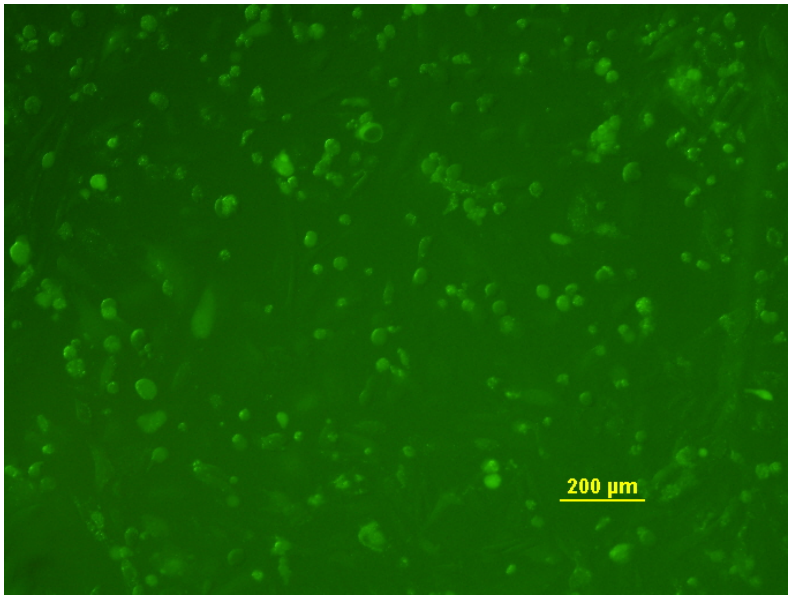
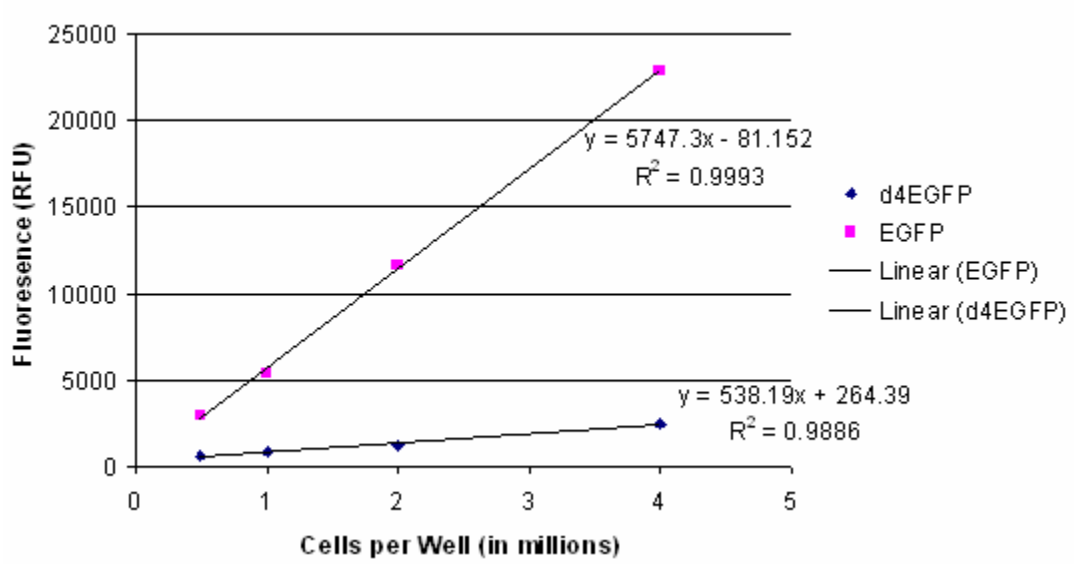


Figure 1. The clonal cell line established exhibits fluorescence after many passages. The intensity differs among cells which may be due to differences in expression at different cell phases.

4.2 Fluorescence signal and cell number correlation

In both a medium suspension and in 3D scaffolds fluorescence had a statistically significant correlation (at $\alpha = 0.5$) with number of cells for both d4EGFP and EGFP expressing cells (see Figure 2). It was expected, based on the differences in half lives and assuming similar expression levels, that around 6 times more fluorescence protein would accumulate in each cell for EGFP as compared to d4EGFP. However, in medium suspension, the slope of the fluorescence with respect to the EGPF expressing cell number was 10.7 times higher than the slope with respect in the d4EGFP expressing cell number. The discrepancy from the expected 6-fold difference to the observed 10.7-fold difference could indicate that the expression level of the d4EGFP cell line is lower than that of the EGFP cell line.



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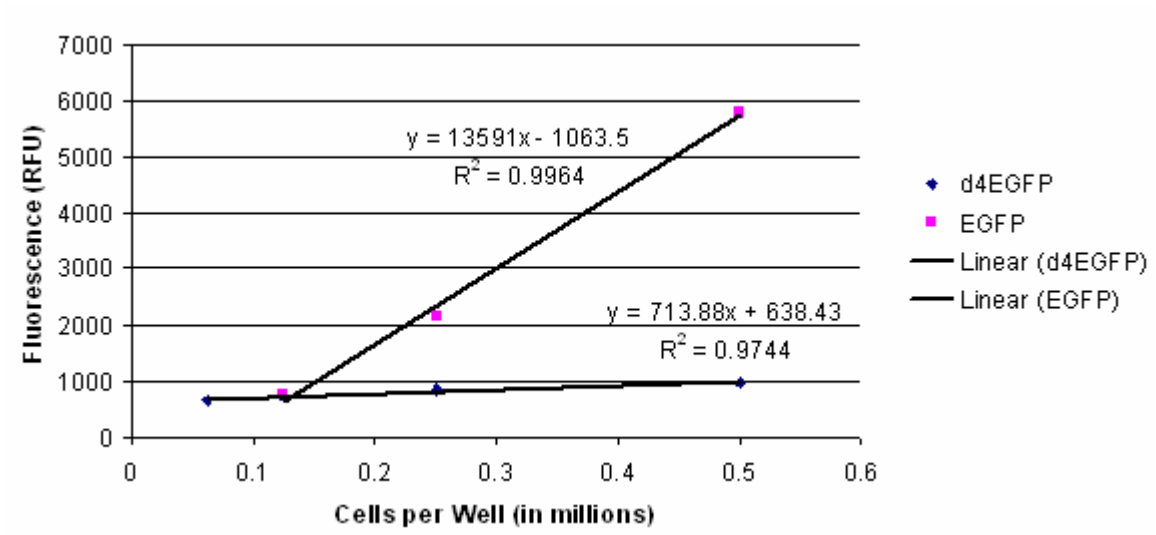

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Figure 2. In both the 2-D culture (A) and in the 3D scaffolds (B), the fluorescence varies linearly with number of cells.

The slopes with 3-D scaffolds were higher than those in suspension, a result obtained in previous work in the group (Zhang, 2008); however, this effect was less pronounced than previously observed. There was a 2.4 times steeper slope from 3-D scaffolds to medium suspension for EGFP expressing cells and a 1.3 times steeper slope for d4EGFP expressing cells.

The slope obtained from the fluorescence versus cell number indicated that the change in cell number needed to have a change in fluorescence response ~5 units for the 2-D culture would be 10000 cells for d4EGFP and 1000 cells for EGFP. To get the same change in fluorescence response in 3-D would require a change in cell number of around 7000 cells for d4EGFP and 400 cells for EGFP.

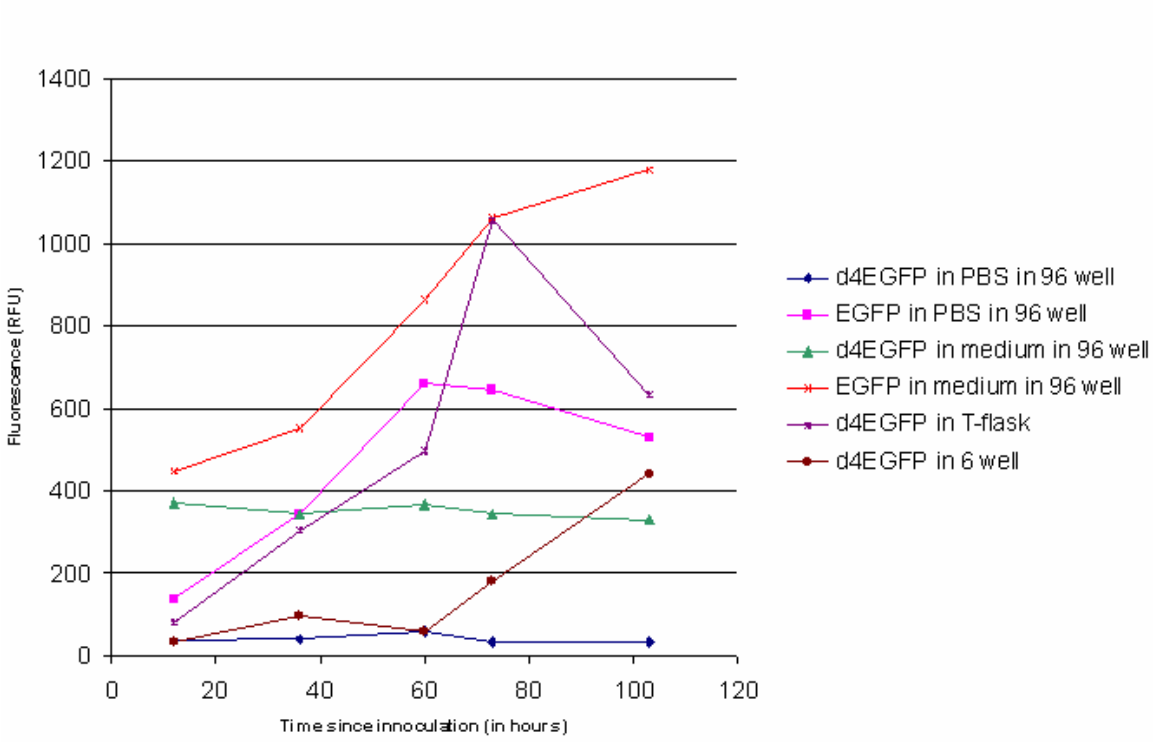


Figure 3. Fluorescence over time had little variation for d4EGFP cells in the 96 well plate. Fluorescence over time had a higher variation for EGFP and for d4EGFP in larger quantities (6 well plate and T-flask).

4.3 Growth kinetics

The change in fluorescence response over the course of the growth kinetics experiment is summarized in Figure 3. The d4EGFP expressing cells had a low range of fluorescence response in the 96 well plate, with EGFP expressing cells in the 96 well plate having around 20 times the range of response. The difference in response range is

due in part to the difference in cell growth (see Figure 4). The range in cell number count of EGFP expressing cells was about 4 times that of d4EGFP expressing cells. The fluorescence range for d4EGFP cells from the 6 well plate and from the T-flask were higher and could show a change in cell number more clearly; however, this required removing the cells from their source, centrifuging and placing them in a 96 well plate to measure the fluorescence.

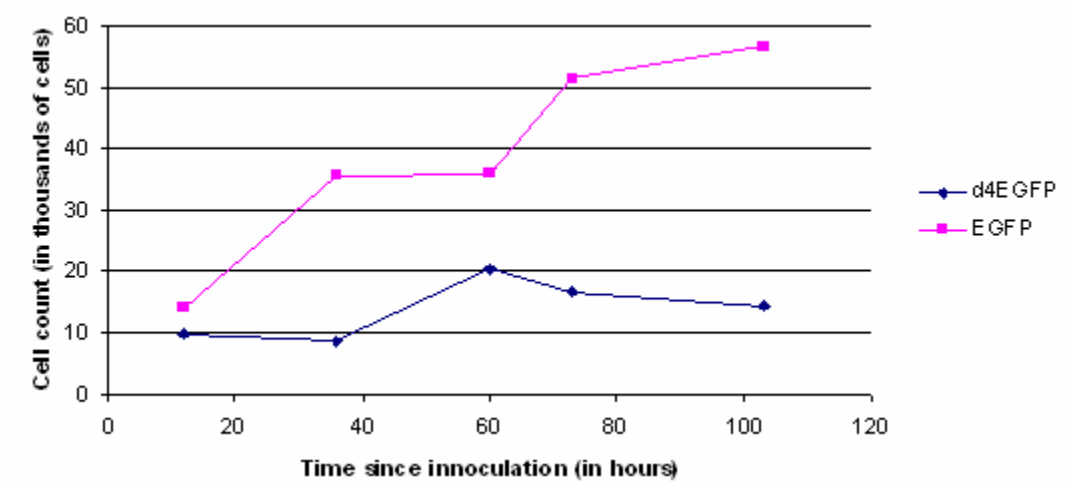
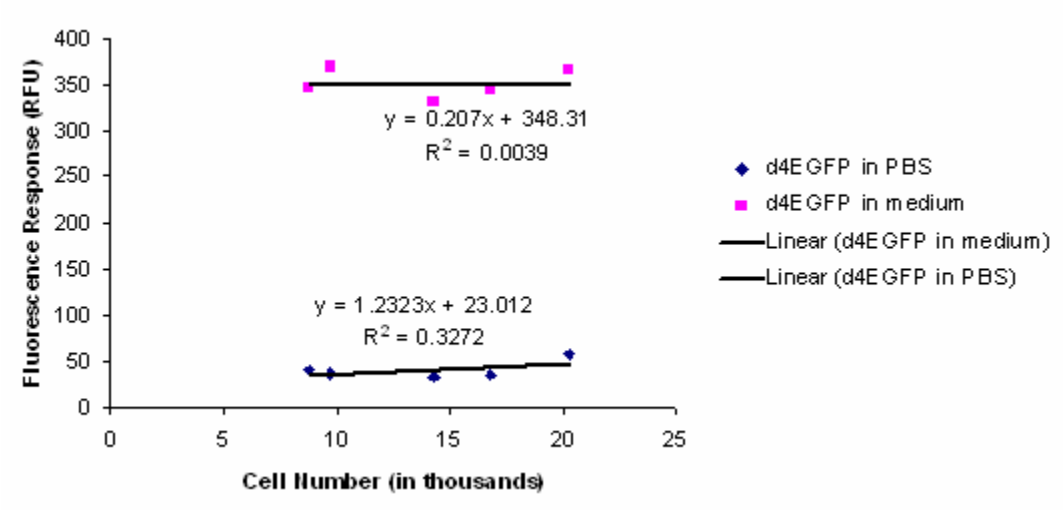


Figure 4. The overall change in cell number for the d4EGFP expressing cells was lower than that of the EGFP expressing cells. The d4EGFP expressing cells decreased in number more quickly than the EGFP expressing cells.

The fluorescence response from the 96 well plate is compared with the cell count from the 96 well plate in Figure 5. At an alpha level of 0.05, the fluorescence response had a significant correlation with cell count for the EGFP expressing cells in medium ($p=0.03$), but not for EGFP in PBS ($p=0.13$), d4EGFP in medium ($p=0.92$) or d4EGFP in PBS ($p=0.31$). The EGFP cells had a closer correlation between cell number and fluorescence than the d4EGFP cells. For EGFP the slopes of the fluorescence versus cell number were higher than predicted from the fluorescence and cell number correlation experiment. For

d4EGFP the slope was similar to what the previous experiment predicted but was too low to have a significant change in fluorescence.



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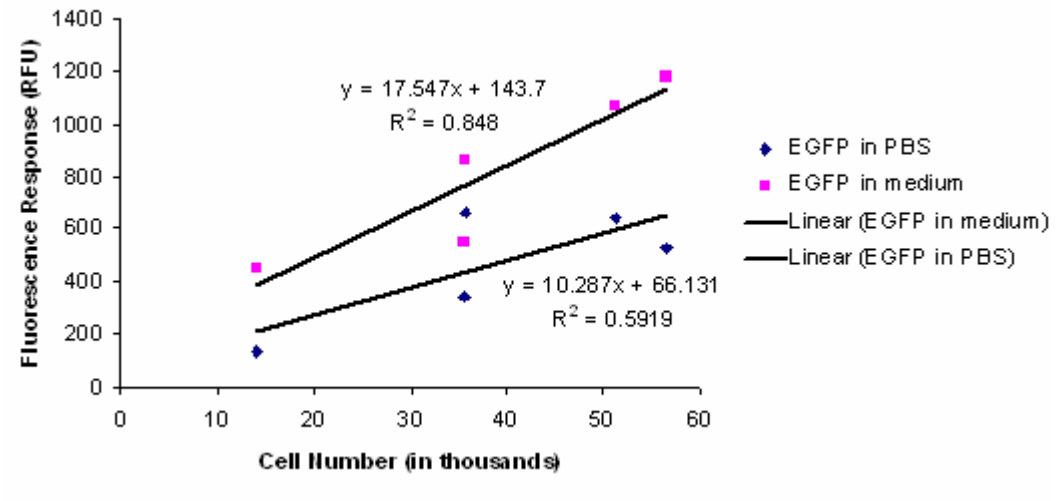


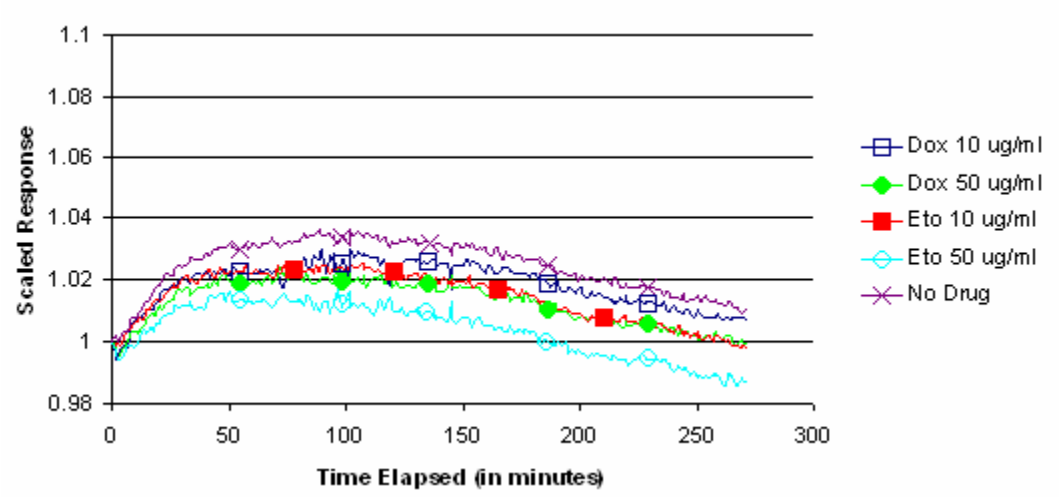
B

Figure 5. The correlation between fluorescence and cell number for d4EGFP expressing cells (A) and EGFP expressing cells (B). EGFP cells had a better overall correlation (higher R^2) and a larger relative response (slope of line).

4.4 2-D toxicity in PBS suspension

The toxicity experiment indicated that the fluorescence changes in response to drug addition (Figure 6). An unexpected result was the increase in fluorescence that was

observed initially with EGFP expressing cells and during two periods with d4EGFP expressing cells. It is suspected that this might be due to recovery from photobleaching or oxidation of the fluorescence proteins. The trend of fluorescent response is as expected, with fluorescence response changing due to drug exposure in a dose dependent manner. The response is more discernable with the EGFP expressing cells than with the d4EGFP expressing cells.



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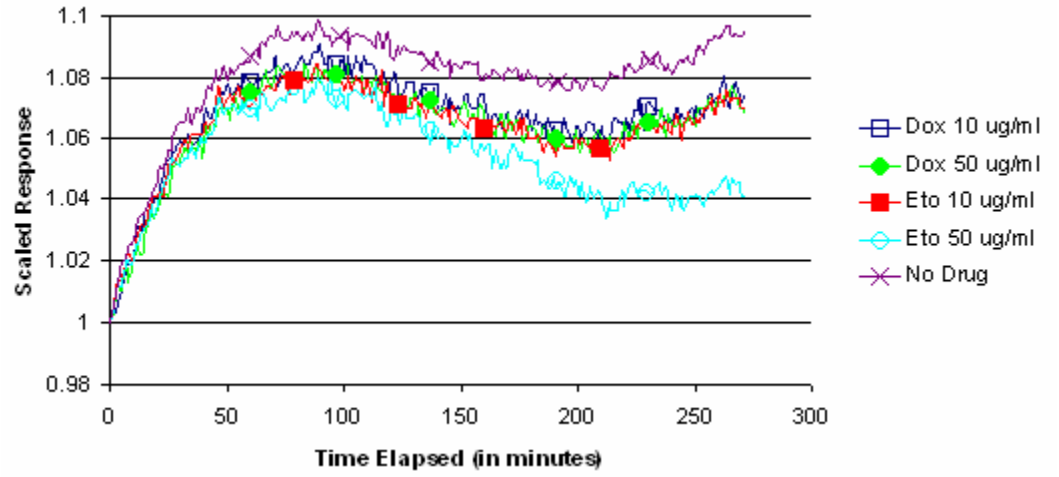
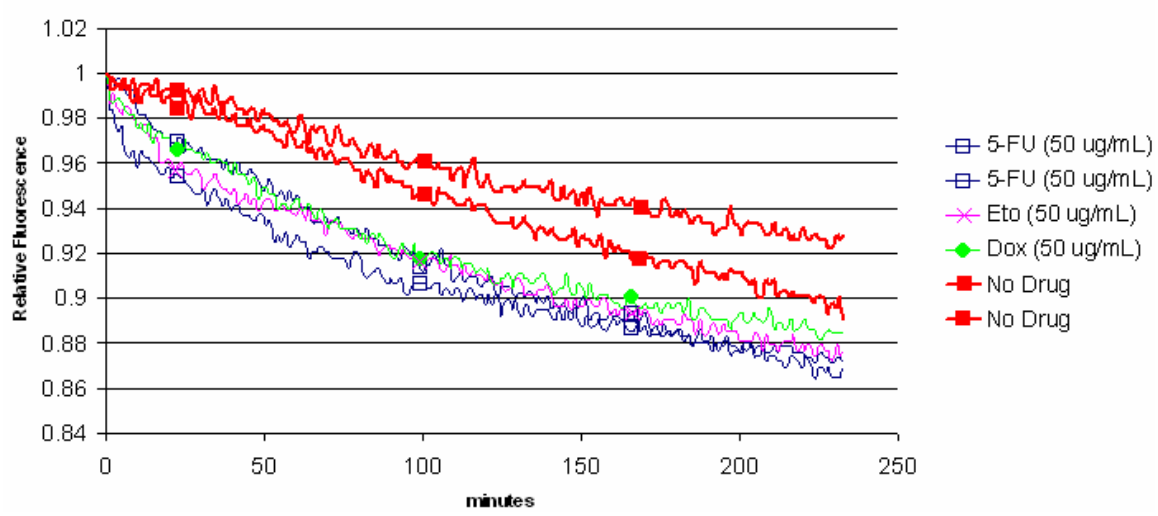


B

Figure 6. The change in fluorescence response for EGFP expressing cells (A) and d4EGFP expressing cells (B) was highest with no drug and lower with drug, with the most reduced scaled response for etoposide at 50 µg/ml.

4.5 2-D toxicity with anchored cells

In a second toxicity test cells were assayed in both PBS and medium as shown in Figure 7. As occurred with the growth kinetics experiment the cells in medium had a larger magnitude of fluorescence change than the cells in PBS indicating that the cells' interaction with the medium affects the fluorescence along with the d4EGFP. For the trials performed in medium the fluorescence response was similar for each drug and depressed the fluorescence as compared with wells with no drug added. For the trials in PBS the fluorescence change was positive and was roughly the same for all trials with the doxorubicin trial having a slightly lower change. It is possible that the drug does not act quickly enough on the cells to result in a significant change in fluorescence which would be why there was not a significant difference in fluorescence change due to the addition of drug.



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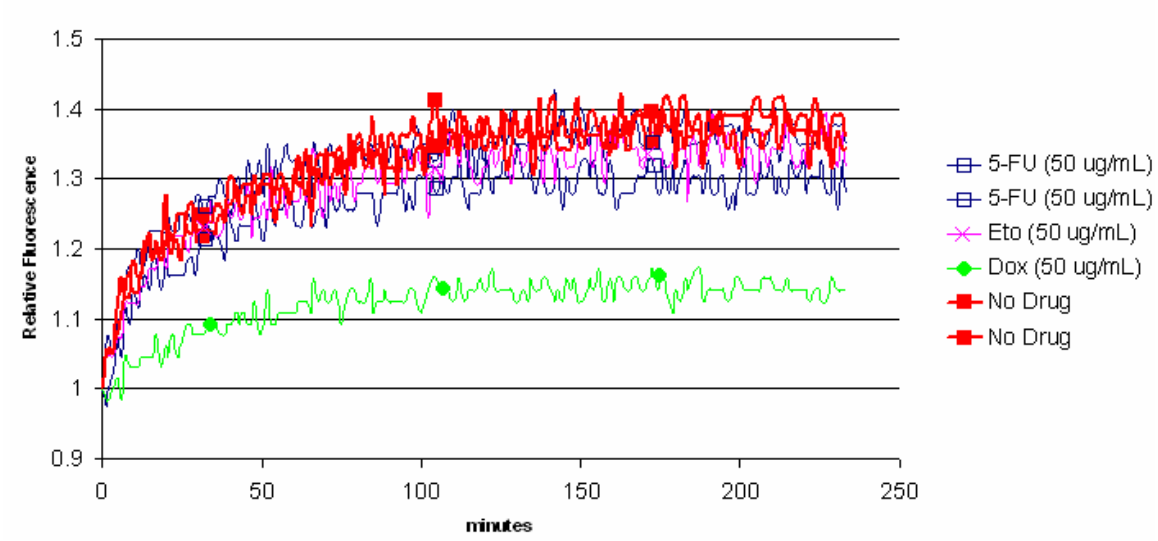

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Figure 7. The fluorescence change observed with cells suspended in medium (A) was similar with all the drugs used and was lower than with no drug. The fluorescence change with cells suspended in PBS was similar for all treatments with the exception of a slightly lower change with doxorubicin.

Chapter 5. Conclusions and Recommendations

The d4EGFP expressing cell line may not be suited for high-throughput screening from 2-D culture using fluorometry due to the low intensity of fluorescence per cell. Fluorescence per cell for 3-D was slightly improved from 2-D but more work is needed to determine whether the cell line can suitably report fluorescence for high-throughput 3-D cellular assays. Since previous work has shown that higher cell densities can be obtained in 3-D culture it is still possible that the cell line will be able to have a significant enough fluorescence change in response to changes in cell number. Growth kinetic experiments using the 3-D culture would shed light on this feasibility.

Since some of the fluorescence changes were not as expected for the experiments, future work should also consider the effect of environmental factors such as photobleaching, oxygen levels, pH and temperature to determine whether fluorescence might have been affected in some unexpected way by such factors. If such factors do significantly affect fluorescence then they might be able to be better controlled for future experiments and allow for better results.

If ultimately the d4EGFP expressing cell line proves unsuitable for cell number reporting, then there is still a possibility that future transfection attempts could yield a cell line with higher fluorescence expression. It may be that the location of the plasmid insert is responsible for the low levels of fluorescence expression. Future transfection experiments might yield cell lines with gene inserts in a region with higher expression. Also, it is possible that the current cell line could be transfected again and sorted by fluorescence intensity to yield a cell line that has multiple copies of the fluorescence protein gene and would thus have improved expression.

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